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# Incoming nucleotide binds to Klenow ternary complex leading to stable physical sequestration of preceding dNTP on DNA

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## ABSTRACT

**Klenow–DNA complex is known to undergo a rate-limiting, protein conformational transition from an 'open' to 'closed' state, upon binding of the 'correct' dNTP at the active site. In the 'closed' state, $Mg^{2+}$ mediates a rapid chemical step involving nucleophilic displacement of pyrophosphate by the 3′ hydroxyl of the primer terminus. The enzyme returns to the 'open' state upon the release of PPi and translocation permits the next round of reaction. To determine whether Klenow can translocate to the next site on the addition of the next dNTP, without the preceding chemical step, we studied the ternary complex (Klenow–DNA–dNTP) in the absence of $Mg^{2+}$. While the ternary complex is proficient in chemical addition of dNTPs in $Mg^{2+}$, as revealed by primer extensions, the same in $Mg^{2+}$-deficient conditions lead to non-covalent (physical) sequestration of first two 'correct' dNTPs in the ternary complex. Moreover, the second dNTP traps the first one in the DNA-helix of the ternary complex. Such a dNTP–DNA complex is found to be stable even after the dissociation of Klenow. This reveals the novel state of the dNTP–DNA complex where the complementary base is stacked in a DNA-helix non-covalently, without the phosphodiester linkage. Further, shuttling of the DNA between the polymerase and the exonuclease site mediates the release of such a DNA complex. Interestingly, Klenow in such a $Mg^{2+}$-deficient ternary complex exhibits a 'closed' conformation.**

## INTRODUCTION

Replication of DNA with very high precision is essential for the survival of any organism. Insights into the fidelity of DNA replication by polymerases have been obtained mostly through studies that involved pre-steady-state kinetics of DNA synthesis (1–8), site-directed mutagenesis (9–19) and high-resolution crystal structure of polymerases (20–26). DNA polymerases achieve this with extremely high fidelity by discriminating between the 'correct' and the 'incorrect' nucleotides in several steps of base insertion. Extensive studies, based on both single turnover and pre-steady-state kinetics, have enabled a clearer understanding of the kinetics of various steps involved in correct base insertion and editing fidelity of Klenow fragment (1–5). First, the binding of a wrong nucleotide is weaker than the correct one (27). Secondly, a rate determining conformational change, from an 'open' to a 'closed' state of the polymerase takes place only upon binding of the correct nucleotide. This in turn positions the 3′-OH of the primer terminus and dNTP for the nucleophilic attack (1–3,28–30). Further, the slower rate of incorporation of nucleotides at a mismatched base pair end allows longer time for the exonucleolytic proofreading to remove the wrong base (4).

Translocation of the polymerase to the next site for the subsequent round of catalysis is not a distinct rate-limiting step. Free diffusion of the polymerase between the '$n$' and '$n + 1$' sites allows rapid equilibration of these two sites. Binding of the dNTP or PPi shifts the equilibrium to either of the sites (31). According to the model proposed by Guajardo and Sousa (32), the binding of correct dNTP at the active site drives translocation. The polymerase can slide with respect to the 3′ end of the primer strand and the relative occupancy of different positions on the template is determined by relative free energy of dNTP binding. Following the chemical step and prior to translocation, 3′ dNMP of primer terminus occupies the site which otherwise would be occupied by the dNMP moiety of original dNTP. In the absence of bound dNTP, this configuration allows the energetically most favored state as it facilitates the largest number of favorable contacts between the polymerase and DNA. Translocation of the polymerase would result in net loss of such interactions between the polymerase and DNA at the dNTP-binding pocket. Therefore, forward translocation of the polymerase is largely favored by the free energy of dNTP binding. Thus, this mechanism proposes that the force exerted during translocation is an exact function of the energy available from dNTP binding and not a fractional function of energy available from dNTP hydrolysis. In view of this, we attempted to understand whether Klenow could translocate to the next site upon the addition of the second 'correct' nucleotide, without the hydrolysis of preceding dNTP. In order to get an insight into these aspects, in the absence of chemical incorporation, we chose to use $Mg^{2+}$-free conditions and analyzed these complexes on a native gel. Here, we show that in the absence of $Mg^{2+}$, the next incoming 'correct' nucleotide traps the first

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dNTP in the DNA-helix, which is stable even after the dissociation of Klenow. This is the first report of such a novel dNTP–DNA complex. Further, we describe the dynamic relationship of such complex vis-à-vis the polymerase–exonuclease site.

## MATERIALS AND METHODS

### Materials

T4-polynucleotide kinase and dithiothreitol (DTT) were purchased from Amersham Life Science. Nucleotide triphosphates (dNTPs) and protein molecular weight markers were purchased from Life Technology Inc. Trypsin was purchased from Sigma. Exo– Klenow, carrying a double mutation, D355A E357A, and proteinase K were purchased from USB. The over-expression strain of Klenow fragment (wild-type) (CJ379) was a kind gift from Dr Catherine Joyce of Yale Medical School (USA). The protein was purified as per the procedure described (33). The oligonucleotides were purchased from Keck Biotechnology Resource Laboratory at Yale University. These were purified by electrophoresis on 12% polyacrylamide gels containing 8 M urea, as described (34). The sample of oligonucleotide was subsequently desalted by passing through a Sep-pak C18 cartridge (34). The purity of oligomers was judged by $^{32}$P-labeling of a small portion by T4 polynucleotide kinase, followed by analysis on a 12% polyacrylamide sequencing gel. The duplex substrates (Oligo A and Oligo B) were generated by annealing the template and primer strands at a molar ratio of 1.1:1.0. In these conditions, the primer strands were found to anneal completely. Oligo B substrate was used only in the experiment described in Figure 2 and Oligo A was used in all experiments. Oligo A: 5′-CAGATTCAGCA-3′; 3′-GTCTAAGTCGT**TA**ATTCGAGAT-5′. Oligo B: 5′-CAGATTCAGCA-3′; 3′-GTCTAAGTCGT**AT**ATTCGAGAT-5′. All DNA concentrations are expressed in terms of 3′-OH of the primer strand.

### Gel retardation assay

The ternary complex was formed by incubating annealed duplex substrate (1 μM) with Klenow fragment (0.6 μM) in the presence of dATP (5 μM containing 10 μCi of [α-$^{32}$P]dATP per 10 μl assay) in a reaction buffer [50 mM Tris–HCl (pH 7.5), 0.1 mM EDTA, 1 mM DTT] at 25°C for 30 min. The effect of other dNTPs on such ternary complexes was examined by premixing them at the specified concentrations (see figure legends) with the first nucleotide (5 μM dATP containing 10 μCi of [α-$^{32}$P]dATP). Complexes were electrophoresed on a 7% native polyacrylamide gel equilibrated with 0.5× TB buffer (0.045 M Tris–borate) at room temperature, followed by autoradiography, without drying the gel to prevent the loss of radioactive nucleotide signal from the complexes during vacuum-suction and heating.

### Trypsin fingerprinting assay of Klenow in the ternary complex

Ternary complexes were formed by incubating duplex DNA (1 μM) with Klenow (0.6 μM) at 25°C for 15 min in the presence of a single nucleotide at the specified concentration (see Fig. 6 legend) in a reaction buffer (12 μl) [50 mM Tris–HCl (pH 7.5), 0.1 mM EDTA, 1 mM DTT]. Limited trypsin digestion was carried out at 15 μg/ml trypsin for 6 s, which was quenched by the addition of 6 μl of stop buffer [0.125 M Tris–HCl (pH 6.8), 6% SDS, 30% glycerol and 20 μg/ml bromophenol blue] (30). Electrophoresis was carried out in a 10% SDS–polyacrylamide gel. The bands were visualized by silver staining.

## RESULTS

Based on common overall architectural features associated with thumb, palm and finger domains of several polymerases, that otherwise differ significantly at the primary sequence level, an elegant unifying hypothesis has been put forward to rationalize the mechanism of polymerase action (35,36). A key feature of the hypothesis has been the description of the two-metal-ion site of the catalytic center in a polymerase (37). Here, we describe experiments where Klenow (exonuclease-proficient)–DNA traps the 'correct' first dNTP into a ternary complex in the absence of $Mg^{2+}$ that prevents chemical incorporation and exonucleolytic degradation. The objective of this study has been mainly to look at the effects of next 'correct' nucleotide on the ternary complex (Klenow–DNA–dNTP).

### Ternary (Klenow–DNA–dNTP) and a novel DNA–dNTP complex formed in the absence of $Mg^{2+}$

In order to carry out the experiments in $Mg^{2+}$-free reaction mixtures, residual $Mg^{2+}$, if any, was excluded from the reaction mixture by the addition of EDTA, just sufficient to prevent the polymerization and the exonucleolytic degradation of the primer terminus. Minimum EDTA required for this was estimated by an EDTA titration, followed by electrophoresis in a denaturing polyacrylamide gel to observe the products (data not shown). All the experiments described below contained such an optimized level of EDTA (100 μM) in the absence of $Mg^{2+}$.

The first substrate (Oligo A) used in our study requires the addition of dATP and dTTP as the first and second nucleotides, respectively, at the 3′ end of primer. The unlabeled DNA substrate, Klenow polymerase and dATP yielded a putative 'ternary' complex as monitored by the first 'correct' nucleotide [α-$^{32}$P]dATP) (Fig. 1A, lane 3). Interestingly, when this reaction was supplemented with the next 'correct' nucleotide (dTTP), the yield of the ternary complex, as monitored by the labeled first nucleotide, increased significantly (Fig. 1A, lane 4). Such an enhancement in the yield of the ternary complex upon the addition of the next 'correct' nucleotide is referred to hereafter as the 'next nucleotide effect'. In addition to this enhancement in the level of the ternary complex, it revealed yet another radioactive species, a complex consisting of labeled dATP and duplex DNA. Such a complex that migrated to the position of labeled duplex substrate is, henceforth, referred to as the 'DNA complex' (Fig. 1A, compare lanes 4 and 6). When either the DNA (Fig. 1A, lane 1) or the Klenow (Fig. 1A, lane 2) was excluded from the reaction, neither the ternary nor DNA complex was formed. This clearly indicated that Klenow, DNA and the correct dNTPs (dATP and dTTP) are all necessary and sufficient for the formation of the ternary complex (Klenow–DNA–dNTP) as well as the DNA complex. Surprisingly, the DNA complex was sensitive to detergent (1% SDS) treatment that denatured the Klenow in the ternary complex (Fig. 1A, lane 5). Thus, the DNA complex that co-migrates with the naked DNA (marker) following its dissociation

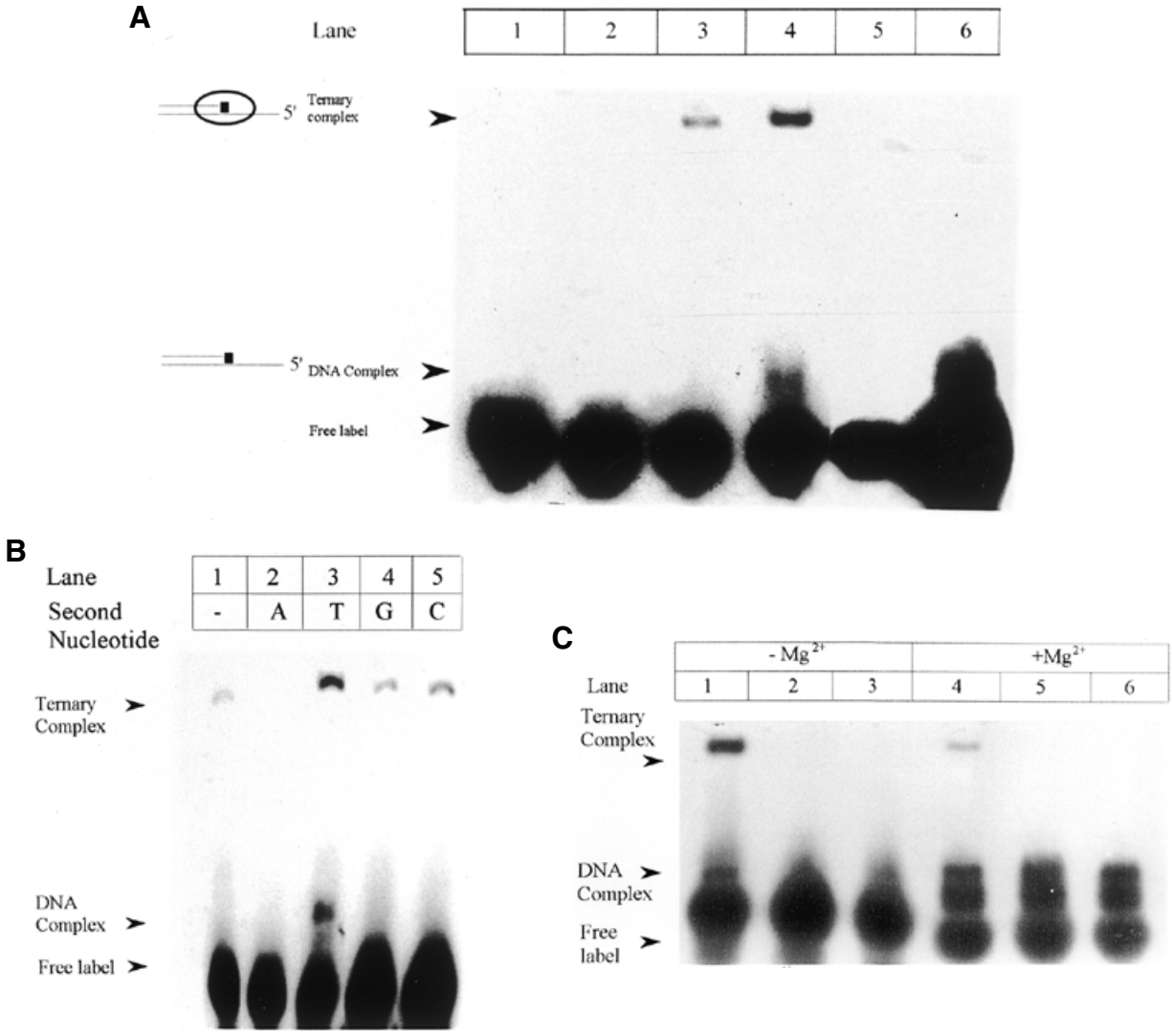


**Figure 1.** Effect of next 'correct' nucleotide on the Klenow–DNA–dNTP ternary complex and the DNA complex: specificity and stability. (**A**) The ternary complex was formed with dATP (5 µM containing 10 µCi of [α-$^{32}$P]dATP) as described in the Materials and Methods (lane 3). A parallel reaction also included unlabeled dTTP (50 µM) (second 'correct' nucleotide) (lane 4). The reaction corresponding to lane 4 was treated with 1% SDS prior to loading on the gel (lane 5). Other controls included: reaction described in lane 4 in the absence of either DNA substrate (lane 1) or Klenow (lane 2); 5′-$^{32}$P-labeled oligomer duplex substrate marker (lane 6). Left, illustrative cartoon diagrams of ternary and DNA complexes are depicted, where the ellipsoid and the filled square represent Klenow and labeled dATP, respectively. (**B**) Ternary complex formed with Klenow, duplex DNA and dATP (5 µM containing 10 µCi of [α-$^{32}$P]dATP) (lane 1) were pre-mixed with either dATP (lane 2), dTTP (lane 3), dGTP (lane 4) or dCTP (lane 5) (each at 50 µM) as unlabeled nucleotide. (**C**) Ternary and DNA complexes are formed by incubating duplex DNA, Klenow, [α-$^{32}$P]dATP (5 µM) and dTTP (50 µM) either in the absence (lane 1) or presence (lane 4) of 10 mM $Mg^{2+}$ as described in the Materials and Methods. The same reactions were treated with either 1% SDS (lanes 2 and 5) or with proteinase K (1 µg/µl) followed by an incubation at 25°C for 20 min (lanes 3 and 6) prior to loading on the gel.

from the ternary complex seems to contain the labeled nucleotide trapped 'non-covalently'.

### Specificity of 'next nucleotide effect'

To assess whether the next nucleotide effect was specific to the 'correct' one (dTTP, in this case), each one of the other dNTPs was tested in the same assay. As observed in Figure 1A, the reaction that contained the first nucleotide (5 µM, [α-$^{32}$P]dATP) yielded only the ternary complex (Fig. 1B, lane 1). The yield of the same was enhanced, with the concomitant release of the DNA complex upon the addition of dTTP as the second nucleotide (Fig. 1B, lane 3). Parallel reactions that contained either dGTP or dCTP as the second nucleotide revealed ternary complexes whose levels were very similar to the control that contained only the first nucleotide and no DNA complex was detected (Fig. 1B, lanes 4 and 5). In yet another reaction, where the equivalent amount of unlabeled dATP was pre-mixed, the ternary complex was not observed (Fig. 1B, lane 2), which was expectedly a result of the dilution of the label. Thus, these experiments reveal that an increase in the steady-state level of ternary complex, as well as the generation of the DNA complex, required the 'correct' next nucleotide.

### DNA complexes versus $Mg^{2+}$ status in the reaction

In order to comprehend the nature of the DNA complex, we compared it with that formed in the presence of $Mg^{2+}$. Under

$Mg^{2+}$-deficient conditions, the first nucleotide ([α-$^{32}$P]dATP) is likely to be trapped non-covalently by the incoming second nucleotide (dTTP). If this hypothesis is correct, a comparison of products formed in the presence of $Mg^{2+}$ ought to reveal a DNA band where the label should be resistant to SDS/proteinase K treatments because of its chemically incorporated status. In the reactions that contained no $Mg^{2+}$, the DNA complexes were sensitive to the SDS/proteinase K treatments that inactivated Klenow (compare lanes 2 and 3 with lane 1 in Fig. 1C). In contrast, the DNA band formed in the presence of $Mg^{2+}$ was completely resistant to SDS/proteinase K treatment (Fig.1C, lanes 4–6). These observations point out that the incoming nucleotide physically traps the first dNTP on the DNA in $Mg^{2+}$-deficient conditions. This has been confirmed by comparing the extension products of the reaction on a denaturing gel. As expected, the $Mg^{2+}$ reactions contain the extension products in addition to the dAMP released as a result of the exonuclease activity of exo+ Klenow. In contrast, $Mg^{2+}$-free reactions showed merely the release of [α-$^{32}$P]dATP (data not shown). Such a resolution between dNMP and dNTP has been reported earlier in denaturing gel conditions (38). Thus, the samples devoid of $Mg^{2+}$ show no chemical incorporation and, hence, demonstrate the physically sequestered state of the label in both the ternary complex and the DNA complex.

### Does the second nucleotide bind to the ternary complex?

Although the addition of the next 'correct' nucleotide enhances the steady-state level of the ternary complex (Fig. 1A and B), it is not obvious if this is because of the binding of the next nucleotide. In order to analyze this, another DNA substrate (Oligo B) was used. Here, the first two bases of the template strand (in Oligo A), namely T and A were flipped (from 3′-TA-5′ to 3′-AT-5′) (bold letters in sequences shown in the Materials and Methods). The resultant Oligo B required addition of dTTP and dATP as the first and second nucleotides, respectively. The first nucleotide (unlabeled dTTP) was added at a concentration of 5 μM, as before, and the second nucleotide (labeled dATP) was added at a concentration of 10 μM instead of 50 μM to reduce the effect of dilution of the label. We compared Oligo A substrate (earlier set used in Fig. 1A and B) with that of Oligo B substrate (present one) under these reaction conditions wherein both the reaction mixtures contained dATP (10 μCi of [α-$^{32}$P]dATP per 10 μl assay) as the labeled precursor. As observed earlier (Fig. 1A), Oligo A formed a ternary complex in the presence of 5 μM labeled dATP (Fig. 2, lane 1). The addition of dTTP (10 μM) as the second nucleotide resulted in the increase of ternary complex yield with a concomitant release of the DNA complex (Fig. 2, lane 2). In the Oligo B substrate reaction, addition of the second nucleotide alone (labeled dATP) led to neither the formation of ternary nor the DNA complex (Fig. 2, lane 3). However, when the reaction mixture was supplemented with the first nucleotide (dTTP), a ternary complex formed (Fig. 2, lane 4). Other controls (data not shown), where either dGTP or dCTP was added instead of dTTP, led to no such ternary complex formation in the presence of labeled dATP. This experiment clearly revealed that labeled dATP (second nucleotide) is detected as a component of ternary complex only in the presence of 'correct' first nucleotide. This demonstrates the presence of second nucleotide along with the first one in the ternary complex. However, the second nucleotide was not a part of DNA complex, as no label was detected at the position of DNA complex (Fig. 2, lane 4). Thus, we conclude that the second nucleotide [α-$^{32}$P]dATP in this case) binds to the ternary complex, but is not released along with the DNA complex. All these experiments (Figs 1A and 2) taken together suggest that the DNA complex essentially retains only the first nucleotide (see Discussion).

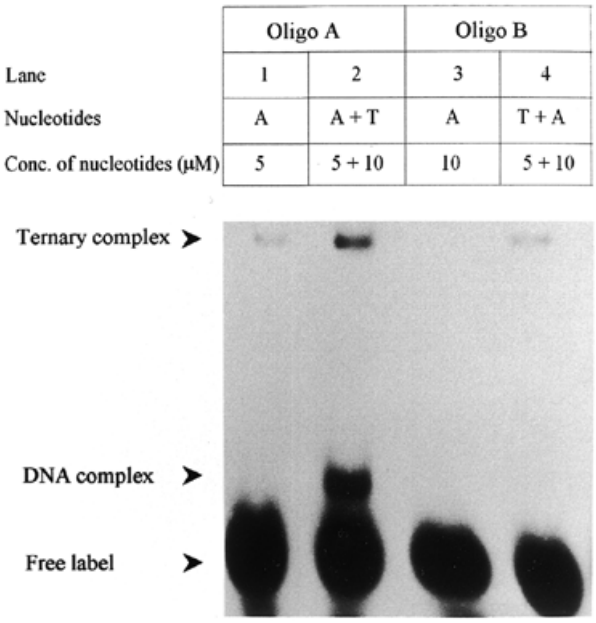


**Figure 2.** Second 'correct' nucleotide is part of the ternary complex, but not the DNA complex. Ternary complexes were formed by incubating Oligo A with Klenow and [α-$^{32}$P]dATP (5 μM) (lane 1). In a parallel reaction, additionally, dTTP (10 μM) was added (lane 2). Similarly, ternary complexes were formed by incubating Oligo B with Klenow and [α-$^{32}$P]dATP (10 μM) (lane 3) to which, additionally, dTTP (5 μM) was added (lane 4). All the samples were analyzed by native PAGE as described in the text.

### The release of DNA complex vis-à-vis the exonuclease domain of the polymerase

What is the molecular basis for the release of the DNA complex? We addressed this question specifically in relation to the twin-active site paradigm of Klenow where polymerase and exonuclease sites are spatially separated far apart (35). Exonucleolytic proofreading model implicates that primer terminus is actively shuttled between these two sites, the partitioning of which seems to critically depend on the 'correctness' of individual bases at the primer terminus (39). To test whether the release of the DNA complex is a result of the large excursion of DNA from the polymerase site to the exonuclease site, we carried out the reaction with exonuclease– Klenow (D355A E357A) (40). Interestingly, the reaction containing dTTP and labeled dATP did not reveal DNA complex with exo– Klenow, even though the ternary complexes were seen (Fig. 3). It is to be noted that the next nucleotide effect manifested only as an increase in the ternary complex and there was no concomitant release of the DNA complex. As the exonuclease activity is not functional in $Mg^{2+}$-deficient conditions used here, it is likely that the observed difference between exo+ and exo– Klenow is a result of differential binding of DNA at the exonuclease site (41). Hence, one

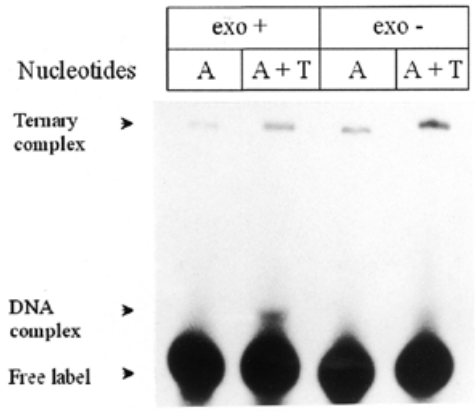


**Figure 3.** The relationship between DNA complexes and the exonuclease domain in the polymerase. Ternary complexes were formed with a polymerase that is either exonuclease proficient (Klenow polymerase) or deficient (Klenow exo–) in the presence of [α-$^{32}$P]dATP (5 μM) and dTTP (50 μM) without $Mg^{2+}$ (see Materials and Methods), followed by native PAGE.

can conclude that the release of DNA complex is a result of partitioning of DNA to the exonuclease site (see Discussion). The exact molecular basis for the same may be clearer upon further analysis of Klenow mutants that are differentially attenuated in polymerase versus exonuclease functions (work in progress).

### Effect of dTTP concentration on the ternary complex and the DNA complex

As increase in the concentration of dNTP is known to reduce exonuclease action (4,42), we monitored the yield of DNA complex as a function of second nucleotide concentration. At a fixed concentration of labeled dATP, the level of dTTP was varied in the reaction mixture, followed by the native gel analyses of the complexes. This revealed dTTP-dependent changes in the level of ternary as well as DNA complexes (gel data not shown). The relative level of labeled dATP associated with either complex was quantitated and expressed as a percentage of total radioactivity in each lane. The ternary complex reached its optimum by the first concentration of dTTP (10 μM tested) and remained so at all concentrations (Fig. 4A). However, in contrast, the DNA complex level increased marginally until dTTP reached 30 μM, followed by a gradual decrease at higher concentration of dTTP. The result seems to point out that the stable sequestration of dATP into the DNA complex depended on an optimum level of next correct nucleotide. As a control, to compare the effect of incorrect nucleotide, we formed the ternary complex with dTTP (50 μM) and labeled dATP (as before) that was subsequently challenged with increasing concentration of dCTP. Increase in concentration of dCTP led to the decline in the level of DNA complex, as expected, without any significant change in the level of ternary complex (Fig. 4B). These results point out a reciprocal relationship between dNTP effect that favors partitioning of the DNA away from the exonuclease site and the release of the DNA complex (see Discussion).

### Klenow exhibits markedly reduced affinity to DNA in the binary as well as the ternary complex formed in the absence of $Mg^{2+}$

The $Mg^{2+}$, in addition to catalysis, seems to have a role in anchoring the DNA substrate to the active site via metal-ion-mediated coordination. This implies that the affinity of DNA to Klenow in $Mg^{2+}$-free ternary complexes would be low. The affinity of labeled DNA to Klenow in such a complex was measured by a gel shift-assay. Binary complexes were studied using end-labeled duplex DNA substrate, titrated with increasing amounts of Klenow, followed by native PAGE. In parallel, we repeated the binding analysis of DNA in the ternary complex by adding unlabeled dATP, the 'correct' dNTP that facilitates the formation of the ternary complex. The radioactivity associated with bands corresponding to the binary/ternary complex and un-complexed DNA was quantitated using a

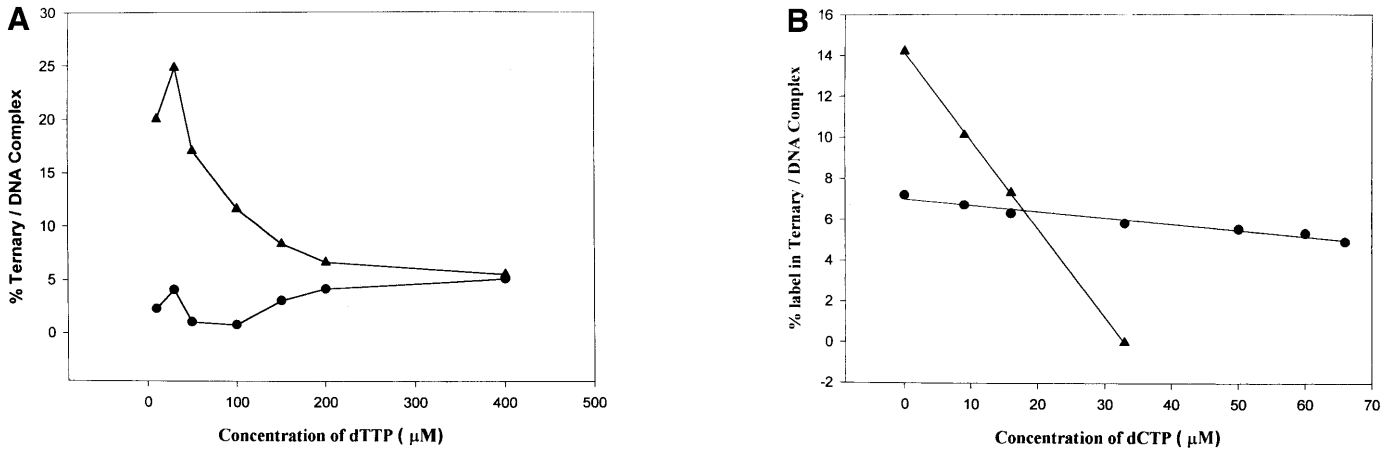


**Figure 4.** Effect of dTTP and dCTP concentration on ternary and DNA complex. (**A**) Ternary complexes were formed by incubating duplex DNA with Klenow, [α-$^{32}$P]dATP (5 μM) and increasing concentrations of dTTP (10–400 μM), followed by native PAGE. (**B**) Ternary complexes formed by incubating duplex DNA with Klenow, [α-$^{32}$P]dATP (5 μM) and dTTP (50 μM) were challenged with increasing concentration of dCTP (0–66 μM) at room temperature for 5 min, followed by native PAGE. Radioactivity associated with ternary complexes, DNA complexes and free label were quantitated using a phosphorimager. Radioactivity associated with ternary (circles) and DNA complexes (triangles) were expressed as percentage of total radioactivity and the mean of three independent experiments is plotted as a function of dTTP (A) and dCTP (B) concentration.

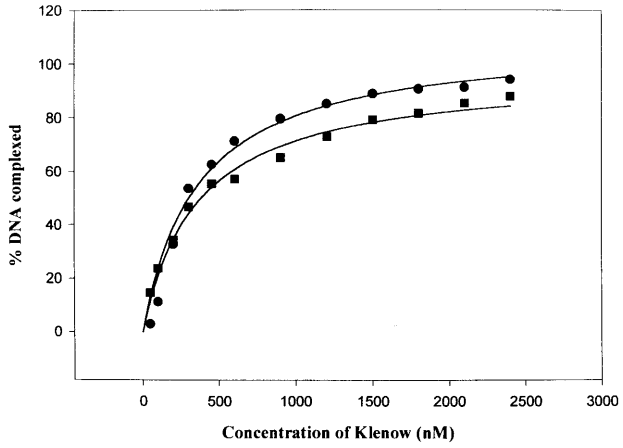


**Figure 5.** $K_{d\,(DNA)}$ analyses of Klenow–DNA complexes (binary and ternary) formed in the absence of $Mg^{2+}$. Binary complexes were formed by incubating 5′ end-labeled duplex substrate (5 nM) with increasing amounts of Klenow (0–2400 nM) in the reaction buffer [50 mM Tris–HCl (pH 7.5), 0.1 mM EDTA, 1 mM DTT] on ice for 15 min, followed by analyses on native 8% polyacrylamide gel at 4°C. The gel was dried and autoradiographed. Similar gel-shift analyses were carried out for ternary complexes formed by incubating 5′ end-labeled duplex substrate (5 nM) with increasing amounts of Klenow (0–2400 nM) in the presence of dATP (0.5 mM). Radioactivity associated with gel-shifted binary/ternary complexes and free DNA was quantified using a phosphorimager. Percentage of total radioactivity associated with the binary/ternary complexes (indicated as percentage DNA complexed) was plotted against Klenow concentration. The data points represent a mean of three independent experiments. The binding isotherms were fitted using Sigma-plot program based on which $K_d$ was computed. Binary complex (circle): ternary complex (square).

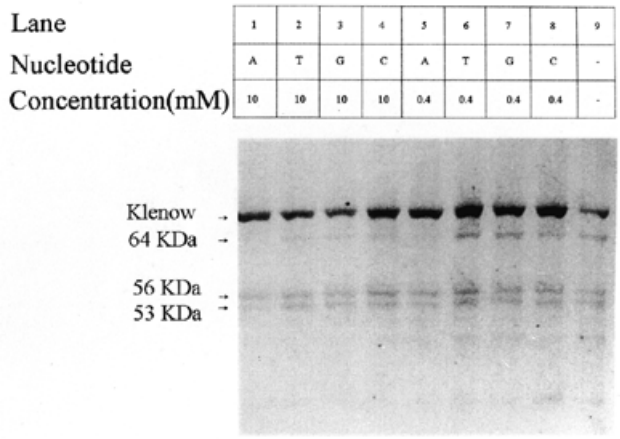


**Figure 6.** Analyses of 'open' and 'closed' Klenow complexes by trypsin fingerprinting assay. Klenow complexes were formed in the presence of the specified nucleotide, followed by trypsin fingerprinting of Klenow (see the Materials and Methods). The samples were analyzed on 10% SDS–polyacrylamide gel, where the fragments were visualized by silver staining.

phosphorimager. Based on this quantitation, binding isotherms of percentage binary or ternary complexes versus Klenow concentration were plotted and the $K_{d\,(DNA)}$ was calculated (Fig. 5). The $K_{d\,(DNA)}$ for $Mg^{2+}$-free binary complex (350 ± 5 nM) was 2–3 orders of magnitude higher compared with a Klenow–DNA complex in the presence of $Mg^{2+}$, as reported earlier (11,43). Formation of a ternary complex, upon the addition of 'correct' dNTP, increased the affinity of DNA merely by ~2-fold [$K_{d\,(DNA)}$ of ternary complex 190 ± 5 nM], which was within the range of enhancement observed for $Mg^{2+}$-containing ternary complexes (43). This analysis suggested an overall drop in the affinity of Klenow to DNA in $Mg^{2+}$-free conditions (see Discussion). We corroborated this using another assay where a pre-formed binary or ternary complex was challenged with increasing concentrations of an unlabeled competitor, a 'trap' DNA. We intended to check whether the DNA in binary versus ternary complexes is differentially sensitive to the trap-challenge, which is indicative of any affinity difference between them. Both the binary as well as the ternary complexes were sensitive to as little as 1–3-fold molar excess of trap-challenge (data not shown). The gel-shifted binary and ternary complexes were equally abolished by 3-fold trap-challenge suggesting that the affinity of Klenow to DNA in either of them is similarly low.

### $Mg^{2+}$-deficient ternary complexes exhibit a 'closed' conformation: trypsin fingerprinting assay of Klenow

The crystal structure of DNA polymerase bound to DNA and dNTP, as well as studies on mutants that abolish metal binding at the polymerase active site implicate $Mg^{2+}$ in anchoring the DNA and the dNTP at the active site (36,37). A recent study on T4 DNA polymerase has demonstrated that the exonuclease site dominates over the polymerase site for possession of the primer terminus, in the absence of $Mg^{2+}$ (44). However, nucleotide triphosphates shift this equilibrium toward the polymerase site. This clearly indicates the binding of the primer strand as well as the dNTP at the polymerase site, even in the absence of $Mg^{2+}$. However, it is not known if Klenow in a $Mg^{2+}$-deficient ternary complex can undergo a rate determining conformational transition from an 'open' to 'closed' state following the binding of 'correct' dNTP. This was analyzed by the partial trypsin digestion experiment of Klenow in ternary complexes. An earlier study, involving such an approach, has revealed that partial trypsin digestion yields a 64 kDa fragment as a signature fragment of 'open' complex (30). The 'closed' complex was characterized by the loss of such a 'fingerprint'. However, this biochemical signature is only a qualitative marker of 'closed' conformational population of Klenow molecules. To make such a populational assay more robust, where most of Klenow is driven into ternary complexes, we performed trypsin digestion assays in a reaction mixture wherein the concentration of the first nucleotide was chosen to be higher than that employed in the radioactive assays described above. However, the $Mg^{2+}$-free conditions were retained. Each nucleotide was tested separately to assess the relationship, if any, between 'closed'conformation versus the 'correct' nucleotide. As expected, 'closed' conformation of Klenow was revealed by the loss of the 64 kDa fragment only in the presence of the 'correct' nucleotide (Fig. 6, compare lanes 5 with 6–8). Such a 'closed' conformation was specific only to the ternary complex, as a control that lacked a nucleotide (Klenow–DNA binary complex) showed an 'open' complex (Fig. 6, lane 9). Addition of a nucleotide other than dATP also resulted in 'open' conformation (Fig. 6, lanes 6–8), suggesting nucleotide selectivity in imparting 'closed' conformation of Klenow. Although a 'closed' conformation ensued, when the nucleotides were present at high concentration (10 mM),

the nucleotide selectivity was relaxed as judged by the relative yield of 64 kDa fragment (Fig. 6, compare lanes 1 with 2–4). These results suggest that the conformation of Klenow is modulated by dNTP in ternary complexes, even in the absence of $Mg^{2+}$. However, the relative yield 53 kDa fragment remained unchanged in 'open' versus 'closed' complexes at all dNTP concentrations tested, which was in contrast to the observations reported earlier for $Mg^{2+}$-containing ternary complexes, where the yield of this fragment was also a reporter of Klenow conformation change (30). This probably reflects additional conformational change occurring in the presence of $Mg^{2+}$. The conformational transitions in Klenow in relation to the added $Mg^{2+}$, DNA and the 'correct' dNTP need a more careful detailed investigation using a real-time fluorescence approach.

## DISCUSSION

Organisms typically tolerate error frequencies of $10^{-9}$ to $10^{-10}$ per base replicated during the duplication of the genome (45). Thus, the high fidelity of polymerase is extremely indispensable for the maintenance of genetic information through generations. This is achieved by the polymerases in several steps of base discrimination prior to and after base incorporation. Structural and energetic differences between Watson–Crick base pairing and incorrect base pairing alone cannot be responsible for such low error rates (46). The geometric constraints imposed by the polymerase active site strongly favor Watson–Crick over non-Watson–Crick structures (47). Studies reveal that selection of the 'correct' base against the 'incorrect' base occurs at varying efficiency at each step of base incorporation. A minimal mechanism for the fidelity of a polymerase action during various stages of base incorporation has been proposed by pre-steady-state kinetics of single turnover events (1–4) and steady-state kinetics typically employing the use of denaturing PAGE (48–50). However, there is very little information on the interaction of the next incoming nucleotide with the ternary complex.

As a result of the $Mg^{2+}$-limiting conditions, the first dNTP is physically trapped in the ternary complex (Fig. 1A and B). On a denaturing gel, such a ternary complex releases no label that is chemically linked to the primer terminus. Upon supplementation with the next nucleotide (dTTP), there was a substantial increase in the steady-state level of the ternary complex. Interestingly, the incoming second 'correct' nucleotide binds to the ternary complex and facilitates the 'next nucleotide effect' described here. Concomitantly, there was a sequestration of first dNTP into DNA-helix, forming the so-called DNA complex, which was stable even after the dissociation of Klenow. Further, the formation of such a novel DNA complex was specific to the addition of 'correct' first and second nucleotides. This is indeed the first report of such physical sequestration of a dNTP in a DNA-helix, an observation that is unprecedented in DNA chemistry. It is important and relevant to comment on the sensitivity of the DNA complex to the treatments that denature Klenow in the ternary complexes. Interestingly, the DNA complex withstands electrophoretic migration through polyacrylamide gel matrix and reveals as a band co-migrating with the duplex substrate (Fig. 1A–C). However, when the binding reaction mixture that contains Klenow–DNA–dNTP ternary complex is treated with either SDS or proteinase K prior to gel analyses, the DNA complex dissociates (Fig. 1A and B). This suggests that any act of 'collapsing' the structure of Klenow in the ternary complex actively disengages the sequestered dNTP from the primer terminus of DNA complex.

Our preliminary evidence suggests a linkage between the release of the DNA complex and the exonuclease domain. Exo+ Klenow released the DNA complex, whereas exo– Klenow did not (Fig. 3). Further, an increase in dNTP concentration that inhibits exonuclease action (4,42), lowers the release of the DNA complex (Fig. 4A and B), which suggests that the exonuclease site has a role to play in the same. In the $Mg^{2+}$-deficient conditions used here, where exonuclease enzymatic activity *per se* is absent, a change in the shuttling frequency of the DNA between polymerase and the exonuclease site, caused by a mutation or increase in dNTP concentration, determines the release of the DNA complex. Elegant studies with T4 DNA polymerase in $Mg^{2+}$-free conditions on equilibrium partitioning of primer terminus reveal a dNTP-dependent switch of DNA from exonuclease to polymerase site (44). According to the model proposed by Galas and Branscomb (51), binding of the next incoming dNTP (correct as well as incorrect) to the polymerase site favors the equilibrium of the primer terminus towards the paired rather than frayed state, which in turn reduces the partitioning of the primer terminus towards the exonuclease site. In other words, this model suggests that the fraction of duplex DNA molecules in a ternary complex susceptible to exonuclease shuttling is reduced as the dNTP concentration is increased. The results describing the effect of increasing concentration of second nucleotide (dTTP or dCTP) are consistent with this hypothesis (Fig. 4A and B). It is interesting to note that within a narrow concentration range of the correct next nucleotide (0–30 μM dTTP), the release of the DNA complex reached an optimum. Any further increase in dTTP/dCTP concentration that reduces shuttling of the DNA towards the exonuclease led to a decline in the yield of the DNA complex.

In the case of the ternary complex formed with [α-$^{32}$P]dATP alone, exonuclease shuttling releases the terminal nucleotide from the complex, which explains why the DNA complex is not detected (Fig. 1A, lane 3). Following the addition of dTTP to the ternary complex, the first nucleotide dATP is stabilized because of the stacking interaction with the 3′-terminal base of the primer strand, which is then released as a DNA complex as a result of exonuclease shuttling (Fig. 1A, lane 4). The second dNTP (dTTP in Oligo A or dATP in Oligo B), which has no chemically anchored base to stack with, is lost from the DNA complex, although it is a part of ternary complex (Fig. 2). The $Mg^{2+}$-free complexes are capable of undergoing a conformational change from the 'open' to the 'closed' state upon binding of the 'correct' nucleotide (Fig. 6). This selectivity for the 'correct' nucleotide was, however, lost at very high dNTP concentration where even the 'incorrect' nucleotides induced this conformational change. Using T4-polymerase in $Mg^{2+}$-free conditions, it was shown that binding of the incorrect dNTP at high concentration shifts the equilibrium partitioning of primer terminus from exonuclease to polymerase site to approximately the same magnitude as the correct dNTP (44). The incorrect dNTP effect at high concentration described there is reminiscent of what we have described in the

experiment where nucleotide selectivity that induced 'closed' conformation was lost at high concentration.

What is the mechanism of the 'next nucleotide effect' observed in this study? How does it stimulate the physical sequestration of the first nucleotide ([α-$^{32}$P]dATP) on the DNA? According to the sliding model of polymerase translocation, binding of either dNTP or PPi can shift the equilibrium of the polymerase between the *n* and *n* + 1 sites (31). Further, Guajardo and Sousa (32) suggest that translocation of the polymerase on the DNA is driven by the binding of next nucleotide. Thus, the binding of the second nucleotide, as described in this paper, drives the translocation of the polymerase on the DNA. In doing so, it 'non-covalently' traps the first dNTP in the DNA-helix. This clearly indicates that chemical incorporation is not a prerequisite step for translocation to ensue. Further, the results reveal that the physical sequestration of the nucleotide ensues in a ternary complex where the affinity of Klenow for DNA is rather low (Fig. 5). It is possible that such a scenario where the DNA is loosely held by a 'closed' conformation of Klenow in the ternary complex is just right for the polymerase to translocate with ease to the next nucleotide. Although the experiments described here suggest the presence of a minimum of two physically sequestered dNTPs in the ternary complexes, it is not clear how far such a 'physical helix' can develop, following unlimited supply of correct dNTPs that are prevented from chemical incorporation? A question that needs careful investigation by using $Mg^{2+}$-limiting protocol described here as well as by employing Klenow mutants that have lost critical amino acids in its active site.

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